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(54) Title: STABLE MICROBUBBLE SUSPENSIONS AS ENHANCEMENT AGENTS FOR ULTRASOUND ECHOGRAPHY

(57) Abstract

Disclosed are injectable suspensions of gas filled microbubbles in an aqueous carrier liquid usable as contrast agents in ultrasonic echography. The suspensions comprise amphipathic compounds of which at least one may be a laminarized phospholipid as a stabiliser of the microbubbles against collapse with time and pressure. The concentration of phospholipids in the carrier liquid is below 0.01 % wt but is at least equal to or above that at which phospholipid molecules are present solely at the gas microbubble-liquid interface. Also disclosed is a method of preparation of the stable suspensions of air or gas filled microbubbles.

STABLE MICROBUBBLE SUSPENSIONS AS ENHANCEMENT AGENTS FOR ULTRASOUND ECHOGRAPHY

Technical Field

The invention relates to injectable suspensions of gas filled microbubbles in an aqueous carrier comprising amphipathic compounds of which at least one is a phospholipid stabilizer of the microbubbles against collapse with time and pressure. The phospholipid stabilizer may be in a lamellar or laminar form. The invention also comprises a method of making stable suspensions of microbubbles usable as contrast agents in ultrasonic echography.

Background of Invention

Use of suspensions of gas microbubbles in a carrier liquid as efficient ultrasound reflectors is well known in the art. The development of microbubble suspensions as echopharmaceuticals for enhancement of ultrasound imaging followed early observations that rapid intravenous injections can cause solubilized gases to come out of solution forming bubbles. Due to their substantial difference in acoustic impedance relative to blood, these intravascular gas bubbles are found to be excellent reflectors of ultrasound. Injecting into the blood-stream of living organisms suspensions of gas microbubbles in a carrier liquid strongly reinforces ultrasonic echography imaging, thus enhancing the visualisation of internal organs. Since imaging of organs and deep seated tissue can be crucial in establishing medical diagnosis a lot of effort is devoted to the development of stable suspensions of highly concentrated gas microbubbles which at the same time would be simple to prepare and administer, would contain a minimum of inactive species, would be capable of long storage and simple distribution. Many attempts towards a solution which will satisfy these criteria have been made, however, none have provided a completely satisfactory result.

form of liposomes i.e. microscopic vesicles, generally spherically shaped. These vesicles are usually formed of one or more concentrically arranged bi-molecular layers of amphipathic compounds i.e. compounds with a hydrophilic and a hydrophobic moieties. The molecules in the bilayers are organised so that the hydrophobic moieties are in facing relationship, the hydrophilic moieties pointing toward the water phase. The suspensions are obtained by exposing the laminarized surfactants to air or a gas prior to or after admixing with an aqueous phase. Conversion of film forming surfactants into lamellar form is carried out according to various liposome forming techniques including high pressure homogenisation or sonication under acoustic or ultrasonic frequencies. The concentration of phospholipids claimed is between 0.01% and 20% and the concentration of microbubbles is between 10^8 and 10^9 bubbles/ml. The microbubble suspensions remained stable for months. The concentration of phospholipids in Example 1 is 0.5%.

An attempt toward a stable echogenic suspension is disclosed in WO-92/11873 (Beller et. al.). Aqueous preparations designed to absorb and stabilise microbubbles for use as an echographic contrasting agent are made with polyoxyethylene/polyoxypropylene polymers and negatively charged phospholipids such as phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine as well as their lysoforms. The concentration range of phospholipids in the preparations may be between 0.01% and 5% by volume or weight, however, preparations with 1% of dipalmitoylphosphatidyl glycerol (DPPG) are specifically disclosed and claimed. In addition to the negatively charged phospholipids the compositions must contain between 0.1% and 10% of polymeric material (Pluronic® F-68). The total amount of solutes in the preparations is between 5.1% and 10.4%. The concentration of the microbubbles is not reported, however, according to the results given it may be estimated to be about 10^7 bubbles/ml. The stability of the suspensions is reported to be better than that of EP-A-0 077 752.

Although the prior art compositions have merit, they still suffer several drawbacks which hamper their practical use. Firstly,

hidden in the fact that in the course of the routine experimentation further reduction in concentration of the ingredients never produced suspensions which were stable enough to have any practical use or encourage further tinkering in the lower end of the known range.

Summary of the invention

The present invention is based on the unexpected finding that very stable suspensions of a gas filled microbubbles comprising at least 10^7 microbubbles per millilitre may be obtained using phospholipids as stabilizers even if very low concentrations thereof are employed. The suspensions usable as contrasting agents in ultrasonic echography are obtained by suspending in an aqueous carrier at least one phospholipid as a stabiliser of the microbubbles against collapse with time and pressure, the concentration of the phospholipids being below 0.01% wt. but equal to or higher than that at which the phospholipid molecules are present solely at the gas microbubble-liquid interface.

It was quite unexpected to discover that as negligible amounts of the phospholipid surfactants involved here (used alone or with a relatively small proportions of other amphiphiles) can so effectively stabilize microbubbles. It is postulated that, in the presence of other amphipathic compounds (such as Pluronic®) the mutual cohesion between stabilizer molecules is decreased and formation of monomolecular phospholipid films is inhibited. However, in the absence of large amounts of other amphiphilic agents, the unhindered intermolecular binding forces (electrostatic interaction or hydrogen bonding) between phospholipid molecules are sufficient to ensure formation of stable film-like structures stabilizing the bubbles against collapse or coalescence.

According to the invention, suspensions of high microbubble concentration, high stability, long storage capacity and ease of preparation may be obtained even if the concentrations of surfactants and other additives in the suspensi ns are kept well below the levels used in the state-of-the-art formulations. The amount of phospholipids used in the compositions of the invention

surfactant may be so low without impairing the stability of the gas bubbles.

The suspensions of the invention offer important advantages over the compositions of the prior art not only because of the low phospholipid content but also because the total amount of injected solutes i.e. lipids and/or synthetic polymers and other additives is between 1,000 and 50,000 times lower than heretofore. This is achieved without any loss of microbubble concentration i.e. echogenicity or stability of the product. In addition to the very low concentration of solutes, the invention provides suspensions which may contain only the microbubbles whose contribution to the echographic signal is relatively significant i.e. suspensions which are free of any microbubbles which do not actively participate in the imaging process.

Needless to say that with such low concentrations of solutes in the injectable composition of the invention probability of undesirable side effects is greatly reduced and elimination of the injected agent is significantly improved.

The microbubble suspensions with low phospholipid content of the invention may be prepared from the film forming phospholipids whose structure has been modified in a convenient manner e.g. by freeze-drying or spray-drying solutions of the crude phospholipids in a suitable solvent. Prior to formation of the suspension by dispersion in an aqueous carrier the freeze dried or spray dried phospholipid powders are contacted with air or another gas. When contacted with the aqueous carrier the powdered phospholipids whose structure has been disrupted will form lamellarized or laminarized segments which will stabilise the microbubbles of the gas dispersed therein. Conveniently, the suspensions with low phospholipid content of the invention may also be prepared with phospholipids which were lamellarized or laminarized prior t their contacting with air or another gas. Hence, contacting the phospholipids with air or another gas may be carried out when the phospholipids are in a dry powder form or in the form f a dispersi n of laminarized phospholipids in the aqueous carrier.

When laminarized surfactants are suspended in an aqueous liquid carrier and air or another gas is introduced to provide microbubbles, it is thought that the microbubbles become progressively surrounded and stabilised by a monomolecular layer of surfactant molecules and not a bilayer as in the case of liposome vesicles. This structural rearrangement of the surfactant molecules can be activated mechanically (agitation) or thermally. The required energy is lower in the presence of cohesion releasing agents, such as Pluronic®. On the other hand, presence of the cohesion releasing agents in the microbubble formulations reduces the natural affinity between phospholipid molecules having as a direct consequence a reduced stability of the microbubbles to external pressures (e.g. above 20-30 Torr).

As already mentioned, to prepare the low phospholipid content suspensions of the invention, in place of phospholipid solutions, one may start with dry phospholipids which may or may not be lamellarized. When lamellarized, such phospholipids can be obtained for instance by dehydrating liposomes, i.e. liposomes which have been prepared normally by means of conventional techniques in the form of aqueous solutions and thereafter dehydrated by usual means. One of the methods for dehydrating liposomes is freeze-drying (lyophilization), i.e. the liposome solution, preferably containing hydrophilic compounds, is frozen and dried by evaporation (sublimation) under reduced pressure.

In another approach, non-lamellarized or non-laminarized phospholipids may be obtained by dissolving the phospholipid in an organic solvent and drying the solution without going through liposome formation. In other words, this can be done by dissolving the phospholipids in a suitable organic solvent together with a hydrophilic stabiliser substance e.g. a polymer like PVP, PVA, PEG, etc. or a compound soluble both in the organic solvent and water and freeze-drying or spray-drying the solution. Further examples f the hydrophilic stabiliser compounds soluble in water and the organic solvent are malic acid, glycolic acid, maltol and the like. Any suitable organic solvent may be used as long as its boiling point is sufficiently low and its melting point is sufficiently high to facilitate subsequent drying. Typical organic solvents would be for

ethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), cardiolipin (CL), sphingomyelins. Examples of suitable phospholipids are natural or synthetic lecithins, such as egg or soya bean lecithin, or saturated synthetic lecithins, such as, dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine or diarachidoylphosphatidylcholine or unsaturated synthetic lecithins, such as dioleylphosphatidyl choline or dilinoleylphosphatidylcholine, with saturated lecithins being preferred.

Additives like cholesterol and other substances can be added to one or more of the foregoing lipids in proportions ranging from zero to 50% by weight. Such additives may include other non-phospholipid surfactants that can be used in admixture with the film forming surfactants and most of which are known. For instance, compounds like polyoxypropylene glycol and polyoxyethylene glycol as well as various copolymers thereof, phosphatidylglycerol, phosphatidic acid, dicetylphosphate, fatty acids, ergosterol, phytosterol, sitosterol, lanosterol, tocopherol, propyl gallate, ascorbyl palmitate and butylated hydroxy-toluene. The amount of these non-film forming surfactants are usually up to 50% by weight of the total amount of surfactants but preferably between 0 and 30%. Again this means that the concentration of the various additives in the low phospholipid content suspensions of the invention are in the range of 0-0.05% which is more than one hundred times less than in the compositions known so far.

It should also be mentioned that another feature of the suspensions of the invention is a relatively "high" gas entrapping capacity of the microbubbles i.e. high ratio between the amount of the surfactant and the total amount of the entrapped gas. Hence, with suspensions in which the microbubbles have sizes in the 1 to 5 μm range, it is tentatively estimated that the weight ratio of phospholipids present at the gas bubble-liquid interface to the volume of entrapped gas under standard conditions is between 0.1 mg/ml and 100 mg/ml.

In practice all injectable compositions should also be as far as possible isotonic with blood. Hence, before injection, small

means that, actually, the optimal phospholipid concentration (within the given limits) will be rather dictated by the type of application i.e. if relatively high phospholipid concentrations are admissible, the ideal concentration value will be near the upper limit of the range. On the other hand, if depending on the condition of the patient to be diagnosed, the absolute value of phospholipids must be further reduced, this can be done without adverse effects regarding microbubble count and echogenic efficiency.

An embodiment of the method comprises selecting a film forming surfactant and optionally converting it into lamellar form using one of the methods known in the art or disclosed hereinbefore. The surfactant is then contacted with air or another gas and admixed with an aqueous liquid carrier in a closed container whereby a suspension of microbubbles will form. The suspension is allowed to stand for a while and a layer of gas filled microbubbles formed is left to rise to the top of the container. The lower part of the mother liquor is then removed and the supernatant layer of microbubbles washed with an aqueous solution saturated with the gas used in preparation of the microbubbles. This washing can be repeated several times until substantially all unused or free surfactant molecules are removed. Unused or free molecules means all surfactant molecules that do not participate in formation of the stabilising monomolecular layer around the gas microbubbles.

In addition to providing the low phospholipid content suspensions, the washing technique offers an additional advantage in that it allows further purification of the suspensions of the invention, i.e. by removal of all or almost all microbubbles whose contribution to the echographic response of the injected suspension is relatively insignificant. The purification thus provides suspensions comprising only positively selected microbubbles, i.e. the microbubbles which upon injection will participate equally in the reflection of echographic signals. This leads to suspensions containing not only a very low concentration of phospholipids and other additives, but free from any microbubbles which do not actively participate in the imaging process.

Bubble suspensions were obtained by injecting in each vial 10 ml of a 3% glycerol solution in water (through the stopper) followed by gentle mixing. The resulting microbubble suspensions were counted using a hemacytometer. The mean bubble size (in volume) was 2.2 μm.

Dry weight (mg/ml)	Phospholipid conc. (μg per ml)	Concentration (bubbles/ml)
0.5	8	9.0×10^6
1	16	1.3×10^7
5	81	7.0×10^7
10	161	1.4×10^8

Preparations were injected to rabbits (via the jugular vein) as well as minipigs (via the ear vein) at a dose of 1 ml/5kg. In vivo echographic measurements were performed using an Acuson XP128 ultrasound system (Acuson Corp. USA) and a 7 MHz sector transducer. The animals were anaesthetised and the transducer was positioned and then fixed in place on the left side of the chest providing a view of the right and left ventricles of the heart in the case of rabbit and a longitudinal four-chamber view in the case f the minipig. The preparation containing 0.5 mg/ml dry weight gave slight opacification of the right as well as the left ventricle in rabbits and in minipigs. The opacification, however, was superior with the 1, 5 and 10 mg/ml preparations.

Example 2

Lyophilisates were prepared as described in Example 1 with air (instead of SF_6) in the gas phase. The lyophilisates were then suspended in 0.9% saline (instead of a 3% glycerol solution). Similar bubble concentrations were obtained. However, after injection in the rabbit or the minipig the persistence of the effect was shorter e.g. 10-20 s instead of 120 s. Moreover, in the minipig the opacification of the left ventricle was poor even with the 10 mg/ml preparation.

preparation and still contained 1-1.2 x 10^9 bubbles per ml. The exceptional stability was found very surprising considering the extremely low amount of phospholipids in the suspension.

The experiment described above was repeated on a second batch of microbubbles using a shorter decantation time in order to collect preferably larger bubbles (batch P132). The median diameter in volume obtained was 8.8 µm and the total surface determined with the Coulter counter was 22 x 10^8 µm^2 per 10^8 bubbles. The calculation showed that 6 µg DAPC for 10^8 bubbles would be necessary to cover this bubble population with a monolayer of DAPC. The actual amount of DAPC determined by HPLC was 20 µg per 10^8 bubbles. Taking into account the difficulty of obtaining precise estimates of the total surface of the bubble population, it appears that within the experimental error, the results obtained are consistent with coverage of the microbubbles with one phospholipid layer.

Echographic measurements performed with different washed bubble preparations showed that upon separation the lower phase gives a much weaker echographic signal than the upper phase or a freshly prepared sample. On a first sight this seemed normal as the white layer on the top of the syringe contained the majority of the gas microbubbles anyway. However, as shown in Fig. 1 the bubble count showed a surprisingly high microbubble population in the lower layer too. Only upon Coulter measurement it became apparent that the microbubbles had a size below 0.5 µm, which indicates that with small bubbles even when in high concentration, there is no adequate reflection of the ultrasound signal.

A four fold dilution of the preparation P132 in a 3% glycerol solution was injected in the minipig (0.2 ml/kg). The preparation of washed bubbles containing 2.5 x 10^7 bubbles per ml and 5 µg of phospholipids per ml provided excellent opacification in the left and right ventricle with utstanding endocardial border delineation. Good opacification was also obtained by injecting to a minipig an aliquot of preparation P145 (diluted in 3% glycerol) corresponding to 0.2 µg of phospholipids per kg. Contrast was even detectable in the left ventricle after injection of 0.02 µg/kg.

residues obtained after freeze drying were saturated with SF_6 (see Example 1), then dissolved in distilled water at a concentration of 100 mg dry weight per ml.

Lipid mixture (weight ratio)	Conc. in tert-butanol(mg/ml)	Bubble conc. (x 10^9/ml)	Median diam (µm)
DSPC	2	1.3	10
DAPC/DPPG (100/4)	2	3.8	7
DSPC/Chol (2/1)	6	0.1	40
DAPC/Plur F68 (2/1)	6	0.9	15
DAPC/Palm. ac. (60/1)	2	0.6	11
DAPC/DPPA (100/4)	1	2.6	8
DAPC/Chol/DPPA (8/1/1)	8	1.2	19
DAPC/DPPA (100/4)*	5	2.4	18.

Legend

DAPC = diarachidoylphosphatidyl choline
DSPC = distearoylphosphatidyl choline
DPPG = dipalmitoylphosphatidyl glycerol (acid form)
DPPA = dipalmitoylphosphatidic acid
Chol = cholesterol
Palm. ac. = palmitic acid
Plur F68 = Pluronic ®F-68
* In this experiment, CF_4 was used as gas instead of SF_6

In all cases the suspensions obtained showed high microbubble concentrations indicating that the initial conversion of phospholipids into liposomes was not necessary. These suspensions were diluted in 0.15 M NaCl and injected to minipigs as described in Example 3. In all cases outstanding opacification of the right and left ventricles as well as good delineation of the endocardial border were obtained at doses of 10-50 µg of lipids per kg body weight or less.

Example 6

PEG-2000 (2 g), DAPC (9.6 mg) and DPPA (0.4 mg) were dissolved in 20 ml of tertiary butanol and the solution was freeze dried overnight at 0.2 mbar. The powder obtained was exposed to SF_6 and then dissolved in 20 ml of distilled water. The suspension containing 1.4 x 10^9 bubbles per ml (as determined by

Claims

1. An injectable suspension of gas filled microbubbles in an aqueous carrier liquid, usable as contrast agent in ultrasonic echography, comprising at least 10^7 microbubbles per millilitre and amphipathic compounds at least one of which is a phospholipid stabilizer of the microbubbles against collapse, characterized in that the concentration of the phospholipids in the carrier liquid is below 0.01% by weight while being equal to or above that at which the phospholipid molecules are present solely at the gas microbubble-liquid interface.

2. The injectable suspension of claim 1, in which the concentration of microbubbles per millilitre is between 10^8 and 10^{10}.

3. The injectable suspension of claim 1, in which the concentration of phospholipids is above 0.00013% wt.

4. The injectable suspension of any preceding claims, in which the liquid carrier further comprises water soluble poly- and oligo-saccharides, sugars and hydrophilic polymers such as polyethylene glycols as stabilizers.

5. The injectable suspension of any preceding claim, in which the phospholipids are at least partially in lamellar or laminar form and are selected from lecithins such as phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidyl-serine, phosphatidylglycerol phosphatidylinositol, cardiolipin and sphingomyelin.

6. The injectable suspension of claim 4 or 5, further containing substances affecting the properties of phospholipids selected from phosphatidylglycerol, phosphatidic acid, dicetylphosphate, cholesterol, ergosterol, phytosterol, sitosterol, lanosterol, tocopherol, propylgallate, ascorbyl palmitate and butylated hydroxy-toluene.

7. The injectable suspension of claim 1, 2 or 3, in which the phospholipids are in the form of powders obtained by freeze-drying or spray-drying.

16. The method of claim 12, in which the conversion is effected by sonicating or homogenising under high pressure an aqueous solution of film forming lipids, this operation leading, at least partly, to the formation of liposomes.

17. The method of claim 16, in which prior to contacting of at least partially lamellarized surfactant with air or another gas the liposome containing solution is freeze-dried.

18. The method of claims 16 and 17, in which the water solution of film forming lipids also contains viscosity enhancers or stabilisers selected from hydrophilic polymers and carbohydrates in weight ratio relative to the lipids comprised between 10:1 and 1000:1.

19. A method of preparation of a suspension of air or gas filled microbubbles comprising a film forming surfactant, a hydrophilic stabiliser and an aqueous liquid carrier, characterised by dissolving the film forming surfactant and the hydrophilic stabiliser in an organic solvent, freeze drying the solution to form a dry powder, contacting the powder with air or another gas and admixing said powder with the aqueous carrier.

20. The method of claim 19, in which the hydrophilic stabiliser is polyethylene glycol, polyvinyl pyrrolidone, polyvinyl alcohol, glycolic acid, malic acid or maltol.

21. The method of claim 19 or 20, in which the organic solvent is tertiary butanol, 2-methyl-2-butanol or $C_2Cl_4F_2$.

22. A method of making an injectable suspension of gas-filled microbubbles according to claim 1, which comprises suspending laminarized phospholipids, and optionally other additives, in an aqueous carrier liquid, said phospholipids having been in contact with said gas prior or after being suspended, under conditions such that a concentration of said microbubbles sufficient to provide an echographic respose is formed in the suspension, allowing a portion of said phospholipids to form a stabilization layer around said bubbles and thereafter depleting the carrier liquid of the excess of phospholipids not involved in microbubble stabilization.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 93/02915

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	WO,A,91 15244 (SINTETICA S.A.) 17 October 1991 cited in the application see page 1, paragraph 1 -paragraph 2 see page 2, paragraph 4 see page 4, paragraph 2 -paragraph 3 see page 5, paragraph 3 see page 6, paragraph 3 - page 7, paragraph 1 see page 8, paragraph 2 see page 9, paragraph 2 - page 10, paragraph 2 see page 11, paragraph 3 see page 12, paragraph 1 see page 13, paragraph 3 -paragraph 4; claims 1,11,19 --- -/--	1-22

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Berte, M

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
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